A novel HMGCoA reductase inhibitor.

Field of invention:

The present invention relates to a molecular moiety called "HMGCoA reductase inhibitor". The invention embodies the nucleic acid and amino acid sequences of scorpion protein (JCH2) and to the use of this sequence and the product in the study, prevention and treatment of diseases associated with the use of this JCH2 product and derivatives of this product (both recombinant and synthetic) as well as the assay for the HMGCoA reductase activity.

Background of the invention:

Cholesterol is needed for cell membrane structure and as a precursor for bile acid and steroid hormones synthesis. Cholesterol is also required for cellular signaling pathway. Nevertheless, excess cellular free cholesterol is undesirable. Elevated plasma low density lipoprotein, (LDL)-cholesterol levels and low levels of high density lipoproteins (HDL)-cholesterol are known risk factors for development and progression of atherosclerosis and coronary heart disease (Brunzell and Hokanson 1999; Tribble and Krauss 1993). Several epidemiologic studies have also demonstrated that elevated plasma concentration of triglycerids and low levels of HDL to be a risk factor for the development of coronary heart disease (Gotto, 2002). HMG-CoA reductase (HMGR) plays an important and rate-limiting role in cholesterol biosynthesis. This enzyme is responsible for catalyzing the conversion of HMG-CoA to mevalonate in the cholesterol biosynthetic pathway and is the target of compounds that are very effective in lowering serum cholesterol. Hyperlipidemic patients are treated with cholesterol lowering medications and particularly with inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. Statins are specific inhibitors of HMG-CoA reductase. These drugs have been shown to lower the LDL-cholesterol level in patients and are the most effective and tolerated drugs currently used in the treatment of hypercholesterolemia (Bays et al, 2002). Currently there are five statins, lovastatin, simvastatin, pravastatin, fluvastatin, and atorvastatin available. These drugs lower cholesterol by slowing down the production of cholesterol and by increasing the liver's ability to remove the LDL-

cholesterol present in the blood. Studies have shown that about 20 to 60 percent lowering of LDL-cholesterol levels can be brought about in patients using these drugs. Statins are also known to reduce triglyceride levels and increase in HDL-cholesterol as well as elevate the synthesis of LDL-receptors. However, different types of statins, seem to show significantly different activities either at the same or different doses (Serajuddin *et al*, 1991). They differ not only in their mode of action but also in their potency (Cohen *et al*, 2000), metabolic rate, tissue selectivity/affinity, absorption (Nezasa *et al*, 2002) and duration of action. Nevertheless, all the statins show lower incidence of adverse effects. Apart from their lipid lowering actions, a direct cholesterol dependent activity, statins are proposed to have other important cholesterol-independent or pleiotropic effects including atherosclerotic stabilization, amelioration of endothelial dysfunction, improved coronary artery compliance and prevention of plaque rupture and thrombus formation (Takemoto and Liao 2001; Liao, 2002; Vaughan, 2003).

The objective of this investigation is to determine the lipid lowering potential of the venom components that are known to form a natural cocktail of many useful inhibitors and therapeutic agents. Our results show that a new HMG-CoA reductase inhibitor can be found in a scorpion venom that is commonly available in Asia. This inhibitor is capable of lowering the HMGCoA reductase activity at much lower concentration than the known inhibitors, Simvastatin, while exhibiting some beneficial effects which are different from that of the commonly used statins.

Summary of the invention:

The JCH2 from the crude venom of Chinese scorpion, *Buthus martensii* has been purified by sequential gel filtration followed by reverse-phase high performance liquid chromatography (HPLC). The protein showed higher inhibitory activity than the Simvastatin when tested HMGCoA reductase activity on both rat hepatocytes as well as HepG2 cell purified enzyme. The percentage of HMGCoA reductase activity inhibition of 0.6uM JCH2 (10ug) is similar to the inhibition by 10um Simvastatin (4ug). The protein has been purified to homogeneity and characterized. The cDNAs encoding JCH2 have been cloned from the venom gland (telson) mRNA. The HMGCoA reductase assay has also

been modified and optimized in our laboratory, thus this assay and the inhibitor, its sequences (nucleotide and amino acid) as well as its derivatives become the subject for this patent..

Description of the Figures and Tables

Figure 1: HMGCoA reductase activity of JC and Simvastatin using (a) rat hepatocyte and (b) HepG2 cell enzymes.

Figure 2: Gel filtration and HPLC purifications of venom JCH2 from *Buthus martensii.*

Figure 3: HMGCoA reductase activity of JC, JCH2 (3A) and simvastatin (3B).

Table: Percentage of inhibition of HMGCoA reductase activity by Simvastatin, JC and JCH2.

Figure 4: Nucleotide and deduced amino acid sequence of cDNA encoding JCH2.

Detailed description of the Invention:

HMGCoA reductase activity of venom.

Venom from Chinese scorpion (*Buthus martensi Karsch*) was tested for HMGCoA reductase activity using Simvastatin as a control for inhibtion. Protein concentration of 1-100ug/ml was used. The inhibitory activity was observed for simvastatin as well as for the scorpion venom at 10uM of simvastatin and 10ug of scorpion venom (Figure 1).

Fractionation of *Buthus martensii Karsch* crude venom by gel filtration.

The Chinese scorpion venom was chromatographed on Sepahdex G-50 gel filtration using 10mM Tris-Cl pH 7.6. Figure 2a shows the elution profile of the crude venom. The HMGCoA reductase inhibition was seen for fractions from peak 1, P1 at 10ug protein. Based on this result, P1 fractions (13, 14, 15), were subjected to reverse phase HPLC (RP-HPLC) on a C18 column (Jupiter C18, 5um; 4.6x250mm). The column was equilibrated with 0.1%TFA in H_2O and the protein was eluted using a step gradient of 0-20%B , followed by 20-70%B and finally 70%-100% B (0.05%TFA in 80% acetonitrile; Figure 2b). All the fractions from RP-HPLC were assayed for the HMGCoA reductase activity at 10ug

protein. The H2 fraction showing high inhibitory activity was further purified on RP-HPLC (Figure 2c).

Characterization of JCH2 protein.

The purified native JCH2 was subjected to Mass spectrophotometer analysis. Mass spectrometry analyses were carried out on a Q-TOFF mass spectrophotometer (Micromass UK Ltd). The processing of the mass spectra was performed using the Micromass MassLynx software according to the manufacturer's protocol. The mass was determined to be 16803Da (Figure 2d). The N-terminal amino acid sequence of this protein was determined to be **-DSLSPWNEGDTYYGCQRQTDEFCNKICKLH-** for the first 30 amino acid residues.

Cloning of the JCH2 from *Buthus martensii*.

Total RNA extracted (Chomczynski and Sacchi, 1987) from the telsons, was reversed transcribed (Armugam *et al*, 1997) and amplified using gene specific primers. An approximately 400bp fragment of PCR product was obtained and subcloned. Putative positive clones were subjected to DNA sequencing (Sanger *et al*, 1977; Armugam *et al*, 1997) on an automated DNA sequencer (Model 3100, Applied Biosystems, USA). All clones showed the presence of cDNA encoding JCH2 (Figure 4). The cDNA encoded for a 22 amino acid signal peptide and 72 amino acid residues of the mature protein (Figure 4).

The proteins have been found to be negatively charged with pI value (5.03) corresponding to acidic proteins. Interestingly, the mass of 16kDa (obtained from protein analysis) does not correlate to the mass deduced from cDNA, The mass deduced from the cDNA sequence is 8132Da while the mass of native protein was determined to be 16803Da. The JCH2 cDNA, showed that the JCH2 contains 7 Cys residues. Thus, only 3 disulphide bridges have been predicted for this protein. However the extra Cys residue might be involved in forming a covalent bonded dimer, as observed in the mass analysis (16803Da).

Applications

HMG-CoA reductase (HMGR) plays an important and rate-limiting role in cholesterol biosynthesis. This enzyme is responsible for catalyzing the

conversion of HMG-CoA to mevalonate in the cholesterol biosynthetic pathway and is the target of compounds that are very effective in lowering serum cholesterol. Statins are specific inhibitors of HMG-CoA reductase. These drugs have been shown to lower the LDL-cholesterol level in patients and are the most effective and tolerated drugs currently used in the treatment of hypercholesterolemia (Bays et al, 2002). Studies have shown that about 20 to 60 percent lowering of LDL-cholesterol levels can be brought about in patients using these drugs. Statins are also known to reduce triglyceride levels and increase in HDL-cholesterol as well as elevate the synthesis of LDL-receptors. Currently there are five statins, lovastatin, simvastatin, pravastatin, fluvastatin, and atorvastatin available. However, different types of statins, seem to show significantly different activities either at the same or different doses (Serajuddin *et al*, 1991). They differ not only in their mode of action but also in their potency (Cohen *et al*, 2000), metabolic rate, tissue selectivity/affinity, absorption (Nezasa *et al*, 2002) and duration of action. JCH2 is a potent inhibitor of HMGCoA reductase in comparison to simvastatin, where the percentage of inhibition of 0.6uM JCH2 is similar to that of 10uM simvastatin (Figure 3 and tables). Further analysis (Gene expression and protein profiling) have shown that the JCH2 brings about the inhibitory effect possibly *via* a different pathway than for the existing statins. Hence, this invention could be proved beneficial. Furthermore there appear to be beneficial cholesterol-independent effects of this invention.

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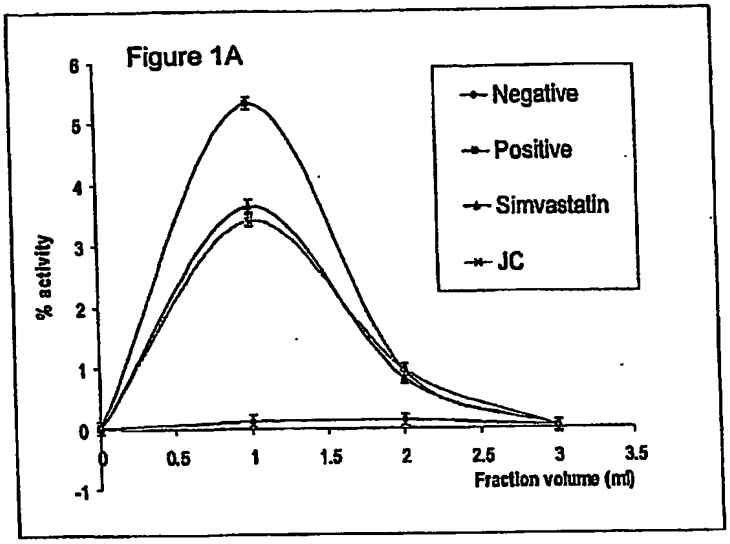


Figure 1A
Negative
Positive
Simvastatin
JC
% activity
Fraction volume (ml)
6
5
4
3
2
1
0
-1
0
0.5
1
1.5
2
2.5
3
3.5

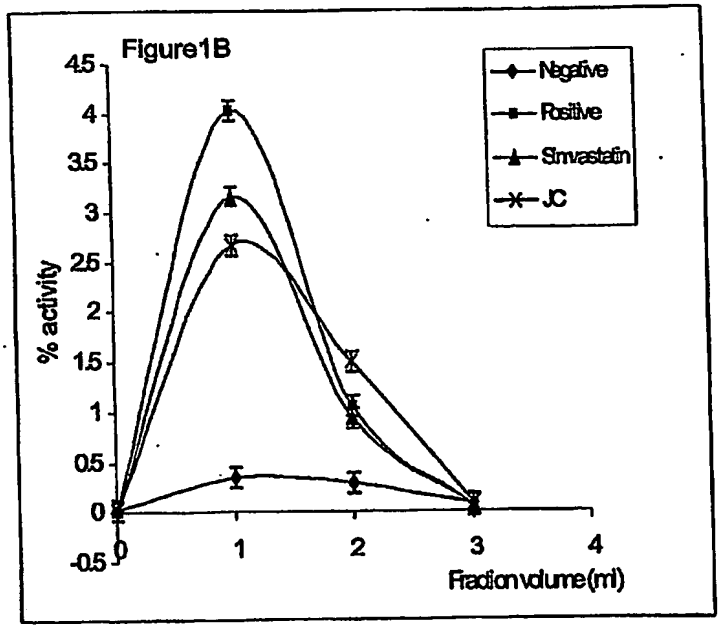


Figure1B
Negative
Positive
Simvastatin
JC
% activity
Fraction volume(ml)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
0
1
2
3
4

Figure 2 A

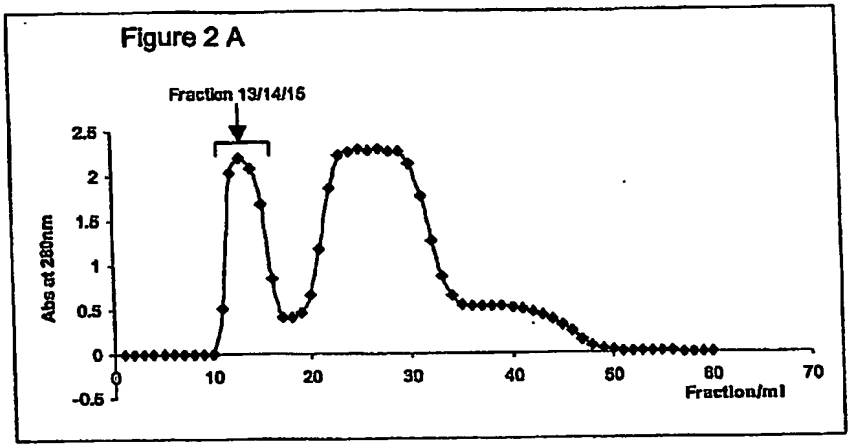


Figure 2B

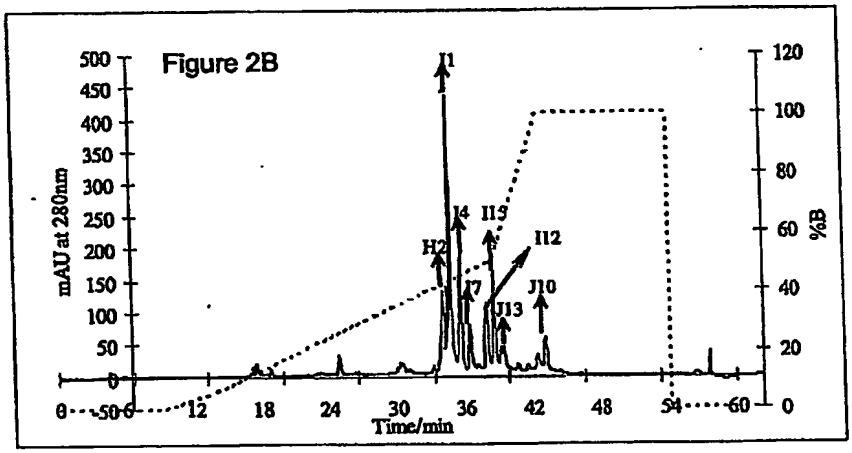
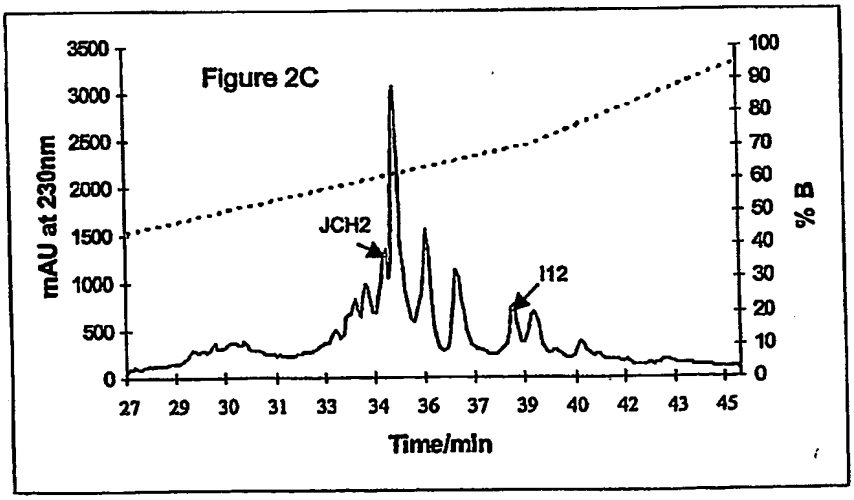


Figure 2C



G2=H2 for HMGCoA reductase inhibition (highest inhibition)

Mass:16803.9Da

Amino-acid sequence:
DSLSPWNEGDTYYGCQRQTDEFCNKICKLH

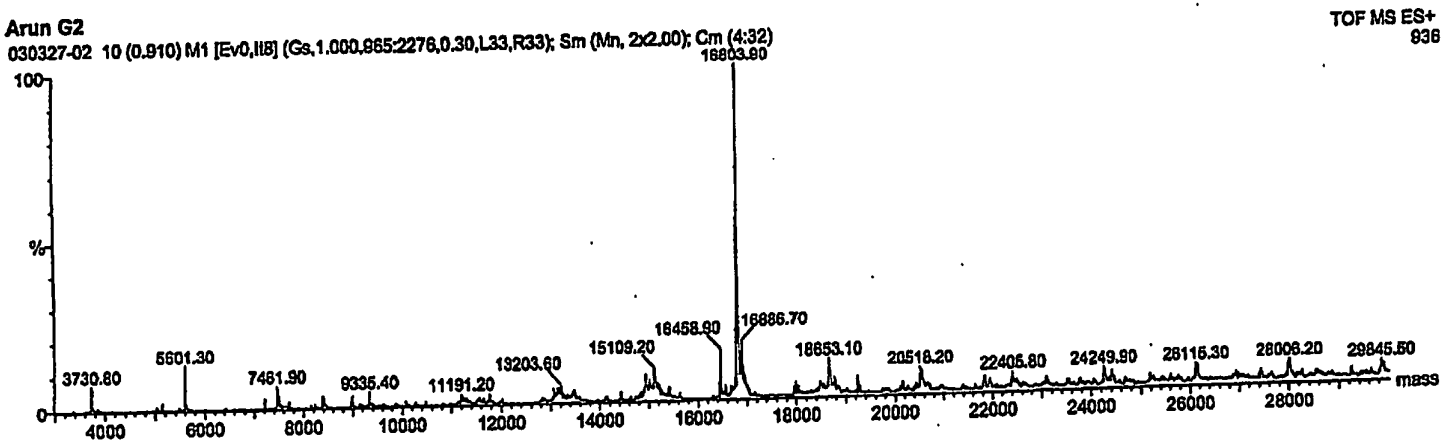
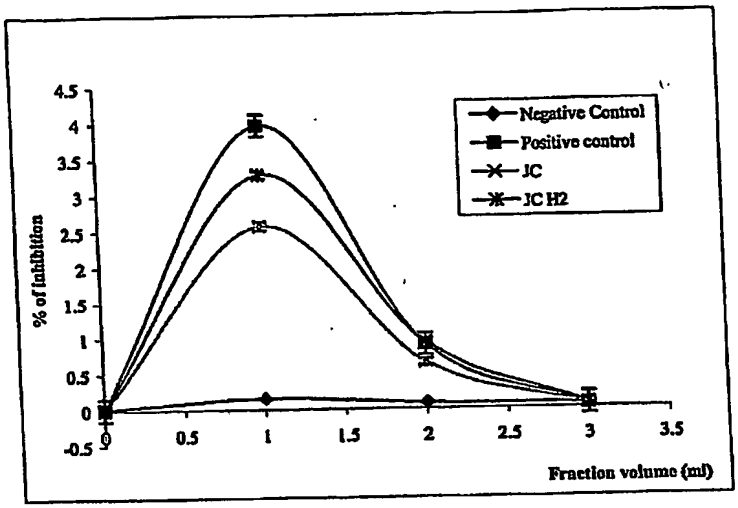
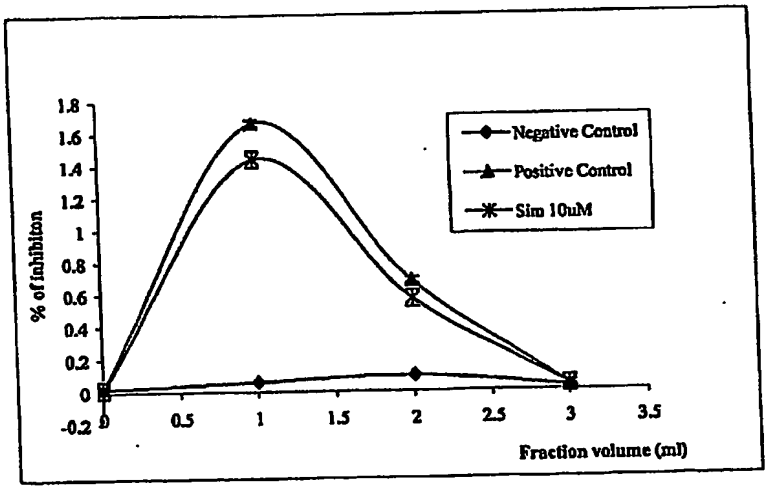


Figure 2d

Figure 3: HMGCoA reductase assay and Table (% of inhibition)



4 hours treatment	% HMGCoA reductase inhibition
Positive Control	0%
JC crude venom (10ug)	35.28%
JC H2 (10ug = ~0.6uM)	17.28%



4 hours treatment	% HMGCoA reductase inhibition
Positive Control	0%
Sim (4ug) 10uM	13.47%

Figure 4: cDNA and deduced amino acid sequence of JCH2 (the N-terminal sequence obtained from direct amino acid sequencing is in bold)

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GGTACATTTCTAAAAAAAGTTATGGTGAAAATGCAAGTTATTTTCATTGCTTTCATCGCTGTA -63
                      M  V  K  M  Q  V  I  F  I  A  F  I  A  V

ATAGCATGTAGCATGGTATATGGAGATAGTCTTTCCCCTTGGAATGAAGGCGATACGTATTAC -126
 I  A  C  S  M  V  Y  G  D  S  L  S  P  W  N  E  G  D  T  Y  Y

GGTTGCCAGAGACAAACGGATGAATTCTGTAATAAAATTTGTAAGCTGCACTTAGCAAGCGGT -189
 G  C  Q  R  Q  T  D  E  F  C  N  K  I  C  K  L  H  L  A  S  G

GGAAGCTGTCAGCAACCCGCTCCTTTTGTGAAATTATGCACATGCCAAGGTATTGATTACGAC -252
 G  S  C  Q  Q  P  A  P  F  V  K  L  C  T  C  Q  G  I  D  Y  D

AACAGTTTCTTTTTTGGAGCATTGGAAAAACAATGTCCTAAATTAAGAGAGTAGCCGAAAGAT -315
 N  S  F  F  F  G  A  L  E  K  Q  C  P  K  L  R  E  *

TTGCATTTATCAATGCTATT -335
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